Effect of day care attendance on sensitization and atopic wheezing differs by Toll-like receptor 2 genotype in 2 population-based birth cohort studies

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Background: Variation in the Toll-like receptor 2 gene *(TLR2/–16934)* is associated with allergic diseases among farmers' children but not among children not living on farms.
Objective: To test the hypothesis that the same genetic variant conferring protection in the farming environment is associated with reduced risk of developing allergic phenotypes among urban children attending day care in early life.
Methods: In 2 population-based birth cohorts (Manchester, United Kingdom, Manchester Asthma and Allergy Study [MAAS]; Tucson, Ariz, Tucson Infant Immune Study [IIS]), participants were recruited prenatally and followed prospectively (MAAS: 3, 5, 8 and 11 years; IIS: 1, 2, 3 and 5 years). We assessed allergic sensitization and atopic wheezing at each follow-up.
Results: A total of 727 children participated in Manchester and 263 in Tucson. We found no significant associations between *TLR2/–16934* and sensitization and atopic wheeze in either cohort. However, a different pattern emerged when we explored the interaction between *TLR2/–16934* and day care attendance on these outcomes. We found a significant interaction between day care and *TLR2/–16934* on the development of sensitization in the longitudinal model in MAAS in that children carrying the T allele who attended day care were less likely to be sensitized than those who did not attend day care, whereas among AA homozygotes, the association tended to be in the opposite direction. In a longitudinal model in IIS, we found a significant interaction between day care attendance and *TLR2/–16934* on the development atopic wheezing. Significant interactions between *TLR2/–16934* and day care were maintained when adjusting for socioeconomic status.
Conclusion: The effect of day care on sensitization and atopic wheezing may differ among children with different variants of the *TLR2* gene. (J Allergy Clin Immunol 2011;127:390-7.)

Key words: *Gene*environment interactions, asthma, allergic sensitization, birth cohorts,* TLR2

Asthma and allergies are the most common chronic diseases in childhood in western societies.[1] Although evidence from twin studies suggests a strong genetic component,[2] there has been little replication among genetic studies.[3] The fundamental role of the environment in the development of these conditions is emphasized by the rapid increase in prevalence that occurred in the last 4 to 5 decades,[1] a time frame too short to be attributable to genetic factors alone. Various environmental exposures have been associated with the development of asthma and allergies. However, as with genetics, the data on the role of environment are often inconsistent, with the same environmental exposure (eg, day care attendance) in different studies conferring an increase in risk,[4] protection,[5-9] or no effect.[10,11] The conflicting evidence on the effect of genetic variants and environmental exposures on allergic phenotypes may be in part a result of the fact that they have largely been studied separately. We propose that the development of sensitization and/or asthma is likely a consequence of environmental factors acting on genetically susceptible individuals through gene-environment interactions. Thus, to understand the role of either genes or environment, it is essential to study both.

The hygiene hypothesis proposes that relative reduction in immune stimulation by microbial exposure consequent to increased hygiene may result in a slower postnatal maturation of the immune system, resulting in higher prevalence of allergies.[12,13] The most convincing evidence for the role of such exposure comes from studies among farmers in central Europe, with a lower prevalence of allergic diseases among farmers' children compared with those not living on farms.[14,15] A recent study in this setting reported that variation in Toll-like receptor 2 gene *(TLR2/–16934, rs4696480)* is strongly associated with the frequency of allergies, and farmers' children carrying a T allele were significantly less likely to have asthma, sensitization, and hay fever compared with children with genotype AA.[16] No such association was found among children not living on farms. Similarly, variations in *TLR2* were shown to modify the associations between country living in childhood and adult asthma in France.[17] In contrast, results from Japan indicated that polymorphisms in

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Abbreviations used
BHR: Bronchial hyperresponsiveness
EVH: Eucapnic voluntary hyperventilation
IIS: Tucson Infant Immune Study
MAAS: Manchester Asthma and Allergy Study
SNP: Single nucleotide polymorphism
SPT: Skin prick test
TLR2: Toll-like receptor 2
UK: United Kingdom

TLRs *are not associated with the development of atopy-related phenotypes.*[18]

Children who attend day care may be exposed to a higher microbial load than those cared for at home[19-21] and consequently have a lower risk of developing allergic phenotypes. However, similar to many other environmental exposures, studies investigating the associations between day care attendance and allergic disease have produced conflicting results.[4-11]

We hypothesized that the same genetic variant conferring a reduction in risk of allergic phenotypes in the farming environment may be associated with a reduction in risk among urban children attending day care in early life. To test this hypothesis, we used data collected prospectively in 2 separate population-based birth cohorts.

METHODS

Study design, setting, and participants

Two population samples were studied (Manchester and Tucson). Both studies were approved by local research ethics committees. Informed consent was obtained from all parents, and children gave their assent if appropriate.

The Manchester Asthma and Allergy Study (MAAS)[22-26] and the Tucson Infant Immune Study (IIS)[27-29] are unselected birth cohort studies (for a detailed description see the Method's section in this article's Online Repository at www.jacionline.org). Participants were recruited prenatally and followed prospectively. They attended review clinics at ages 3, 5, 8, and 11 years (MAAS) and 1, 2, 3, and 5 years (IIS).

Definitions of variables

Day care attendance. In MAAS, "day care" included children who regularly attended day care at any time during the first 2 years of life, and "no day care" included children who were looked after at home or by a child minder.[9] In IIS, "day care" included children who were regularly cared for outside the home at any time during the first 9 months of life.[27]

Sensitization. In MAAS we carried out skin prick tests (SPTs) to common allergens at age 3, 5, 8 and 11 years and defined sensitization as a wheal diameter 3 mm greater than negative control to at least 1 allergen. In addition, we measured specific IgEs at ages 3, 5, and 8 years. In IIS, we measured specific IgEs at ages 1, 2, 3, and 5 years and defined sensitization as specific IgE >0.35 kU_A/L to at least 1 allergen.

Atopic wheeze. Questionnaires were administered to collect information on parentally reported symptoms. Current wheeze was defined as wheeze in the last 12 months and atopic wheeze as current wheeze in the presence of sensitization at the corresponding age.

Airway reactivity (MAAS). Airway reactivity was assessed by eucapnic voluntary hyperventilation (EVH) challenge at age 5 years (see Methods in the Online Repository). Bronchial hyperresponsiveness (BHR) was defined as a change in lung function after challenge greater than the 90th percentile for the reference subjects (skin test–negative, never-wheezing at age 5 years).[30]

Genotyping

Genotyping was performed by using the Single Base Extension method (Sequenom, Hamburg, Germany; MAAS) and 5'-exonuclease assays (Taqman; Applied Biosystems, Carlsbad, Calif; IIS); see Genotyping in the Methods section of this article's Online Repository. For all analyses, AT and TT genotypes were combined to assess our *a priori* hypothesis that the association between day care and sensitization would be evident for children carrying a T allele.

Statistical methods

We used Stata 11.1 (StataCorp LP, College Station, Tex) and SPSS 15.0 (IBM Corporation, Somers, NY) for all analyses. To minimize false-positive results caused by multiple testing and capitalize on the longitudinal nature of the collected data, we made an *a priori* decision to use longitudinal rather than cross-sectional analyses of the 2 phenotypes of interest (sensitization and atopic wheeze) as the primary outcomes. For completeness, the data on a secondary outcome (current wheezing) are presented in the Online Repository (Tables E1 and E2). Longitudinal analyses were performed by generalized estimating equations using the exchangeable correlation structure and the logit link function. We investigated other covariates that might influence clinical outcomes of interest (socioeconomic status, number of siblings, and position of sibship), and models were adjusted as appropriate. For airway reactivity in MAAS at age 5 years, the categoric associations were assessed by using logistic regression models. Only children of European ancestry were included in the analysis.

RESULTS

Participants

In Manchester, we reviewed 1025 children at age 8 years; of those, 122 were randomized to an environmental intervention[31] and excluded from this analysis. Samples for genotyping were provided by 727 white children, of whom 504 attended day care. Of the total IIS population (n = 482), the analyzed sample included 263 white children with data on genotype, day care, and at least 1 outcome. Genotype frequencies were consistent with other populations (AA, 22.0% and 26.6%; AT, 50.9% and 48.7%; TT, 27.1% and 24.7%, MAAS and IIS, respectively); no deviation from Hardy-Weinberg equilibrium could be detected.

Descriptive data

Table I and this article's Table E1 in the Online Repository at www.jacionline.org summarize sex, day care attendance, and clinical outcomes overall and by *TLR2/–16934* in the 2 cohorts. There were no significant associations between *TLR2/–16934* and any clinical outcomes in either cohort. Data on sex and clinical outcomes by day care attendance are presented in Table II and this article's Table E2 in the Online Repository at www.jacionline.org. In MAAS, day care was significantly associated with reduced atopic wheeze at age 8 years (Table II) and reduced wheeze at ages 5 and 8 years (Table E2). In IIS, day care was significantly associated with increased wheeze at age 1 year and reduced sensitization at age 2 years (Tables II and E2). These findings are consistent with our previously reported data.[9,27] In the MAAS cohort, we found that socioeconomic status was significantly associated with day care attendance and some of the outcomes (eg, children from a higher socioeconomic class were more likely to attend day care; in addition, these children were less likely to develop wheeze in early life but more likely to develop sensitization). In IIS, we found no association between socioeconomic status and day care. There was no significant

TABLE I. Sex, day care attendance, and outcomes by *TLR2/–16934* genotype

Variable	Whole group Frequency (%)	AA Frequency (%)	AT + TT Frequency (%)	*P* value*
MAAS				
Population (n = 727)		160 (22.0)	567 (78.0)	
Male	386/727 (53.1)	78/160 (48.8)	308/567 (54.3)	.21
Attended day care	504/727 (69.3)	109/160 (68.1)	395/567 (69.7)	.71
Sensitization (IgE)				
Age 3 y	29/129 (22.5)	5/27 (18.5)	24/101 (23.5)	.58
Age 5 y	116/416 (27.9)	30/96 (31.3)	86/320 (26.9)	.40
Age 8 y	167/414 (40.3)	40/88 (45.5)	127/326 (39.0)	.27
Sensitization (SPT)				
Age 3 y	135/646 (20.9)	33/145 (22.8)	93/501 (20.4)	.53
Age 5 y	165/648 (25.5)	34/142 (23.9)	131/506 (25.9)	.64
Age 8 y	192/657 (29.2)	34/144 (23.6)	158/513 (30.8)	.09
Age 11 y	177/563 (31.4)	38/122 (31.2)	139/441 (31.5)	.94
Atopic wheeze (IgE)				
Age 3 y	11/128 (8.6)	2/27 (7.4)	9/101 (8.9)	1.00
Age 5 y	31/412 (7.5)	9/96 (9.4)	22/316 (7.0)	.51
Age 8 y	53/412 (12.9)	12/87 (13.8)	41/325 (12.6)	.72
Atopic wheeze (SPT)				
Age 3 y	38/642 (5.9)	12/145 (8.3)	26/497 (5.2)	.17
Age 5 y	48/647 (7.4)	12/142 (8.5)	32/505 (7.1)	.60
Age 8 y	69/657 (10.5)	12/144 (8.3)	57/513 (11.1)	.34
Age 11 y	57/563 (10.1)	11/122 (9.0)	46/441 (10.4)	.65
EVH airway hyperreactivity				
Age 5 y	73/473 (15.4)	14/105 (13.3)	59/368 (16)	.50
IIS				
Population (n = 263)		70/263 (26.6)	193/263 (73.4)	
Male	120/263 (45.6)	32/70 (45.7)	88/193 (45.6)	1.00
Attended day care	130/263 (49.4)	34/70 (48.6)	96/193 (49.7)	.89
Sensitization (IgE)				
Age 1 y	26/209 (12.4)	7/57 (12.3)	19/152 (12.5)	1.00
Age 2 y	39/187 (20.9)	13/54 (24.1)	26/133 (19.6)	.55
Age 3 y	57/178 (32.0)	18/50 (36.0)	39/128 (30.5)	.48
Age 5 y	62/153 (40.5)	15/44 (34.1)	47/109 (43.1)	.36
Atopic wheeze (IgE)				
Age 1 y	6/205 (2.9)	2/56 (3.6)	4/149 (2.7)	.67
Age 2 y	10/181 (5.5)	3/52 (5.8)	7/129 (5.4)	1.00
Age 3 y	17/175 (9.7)	5/49 (10.2)	12/126 (9.5)	1.00
Age 5 y	16/146 (11.0)	4/42 (9.5)	12/104 (11.5)	1.00

*χ^2 Test.

association between the number of siblings and position of sibship with exposure of interest and clinical outcomes in either cohort; therefore, these have not been included in the longitudinal models.

Interaction between *TLR2/–16934* and day care

When we explored the interaction between *TLR2/–16934* and day care attendance on clinical outcomes, genotype-specific patterns emerged that were similar in the 2 populations. All estimates for odds ratios and CIs from longitudinal models for allergic sensitization and atopic wheeze are presented in Table III. Significant interactions between *TLR2/–16934* and day care were maintained when adjusting for socioeconomic status; it is of note that adjusting for socioeconomic status did not materially change the odds ratios in these models.

TABLE II. Sex and outcomes by day care attendance

Variable	No day care Frequency (%)	Day care Frequency (%)	*P* value*
MAAS			
Population	223/727 (30.7)	504/727 (69.3)	
Male	112/223 (50.2)	274/504 (54.4)	.30
Sensitization (SPT)			
Age 3 y	47/196 (24.0)	88/450 (19.6)	.20
Age 5 y	51/200 (25.5)	114/448 (25.5)	.99
Age 8 y	62/200 (31.0)	130/457 (28.5)	.51
Age 11 y	52/166 (31.3)	125/397 (31.5)	.97
Sensitization (IgE)			
Age 3 y	13/45 (28.9)	16/84 (19.1)	.20
Age 5 y	33/134 (24.6)	83/282 (29.4)	.31
Age 8 y	55/127 (43.3)	112/287 (39.0)	.41
Atopic wheeze (SPT)			
Age 3 y	12/194 (6.2)	26/448 (5.8)	.85
Age 5 y	15/200 (7.5)	33/447 (7.4)	.96
Age 8 y	28/200 (14.0)	41/457 (8.9)	.05
Age 11 y	19/166 (11.5)	38/397 (9.6)	.50
Atopic wheeze (IgE)			
Age 3 y	5/45 (11.1)	7/84 (8.3)	.61
Age 5 y	10/134 (7.5)	22/282 (7.8)	.90
Age 8 y	25/127 (19.7)	28/285 (9.8)	.01
IIS			
Population	133/263 (50.6)	130/263 (49.4)	
Male	64/133 (48.1)	56/130 (43.1)	.46
Sensitization (IgE)			
Age 1 y	16/110 (14.5)	10/99 (10.1)	.40
Age 2 y	26/94 (27.7)	13/93 (14.0)	.03
Age 3 y	32/88 (36.4)	25/90 (27.8)	.26
Age 5 y	35/82 (42.7)	27/71 (38.0)	.62
Atopic wheeze (IgE)			
Age 1 y	3/109 (2.8)	3/96 (3.1)	1.00
Age 2 y	7/91 (7.7)	3/90 (3.3)	.33
Age 3 y	8/86 (9.3)	9/89 (10.1)	1.00
Age 5 y	9/78 (11.5)	7/68 (10.3)	1.00

*χ^2 Test.

Sensitization

In both cohorts, the effect of day care on sensitization differed by *TLR2/–16934* genotype. In MAAS, in a longitudinal model including skin prick tests from all 4 time points (3, 5, 8, and 11 years), we found a significant interaction between day care and *TLR2/–16934* on the development of sensitization ($P = .05$; Table III). Results did not materially change when sensitization was defined by IgE (Table III; this article's Fig E1 in the Online Repository at www.jacionline.org). For either measure of atopic sensitization and at each time point, children carrying a T allele who attended day care tended to have lower risk of sensitization than those who did not attend day care (Figs 1, *A,* and E1). In contrast, among children with AA genotype, day care attendance

TABLE III. Odds ratios and CIs for the effect of day care on clinical outcomes in longitudinal models, stratified by *TLR2/–16934* (using no day care as a reference group)

	TLR2 = AA		TLR2 = AT/TT		
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value	Interaction *P* value
Sensitization (SPT)					
MAAS, age 3-5-8-11 y	1.8 (0.9-3.6)	.110	0.8 (0.6-1.2)	.278	.05
Sensitization (IgE)					
MAAS, age 3-5-8 y	2.1 (0.9-4.6)	.078	0.9 (0.6-1.3)	.435	.06
IIS, age 1-2-3-5 y	1.3 (0.5-3.1)	.609	0.5 (0.3-0.9)	.027	.10
Atopic wheeze					
MAAS, age 3-5-8-11 y (SPT)	1.2 (0.5-2.8)	.671	0.7 (0.5-1.2)	.170	.34
MAAS, age 3-5-8 y (IgE)	1.2 (0.5-5.2)	.484	0.5 (0.3-0.9)	.016	.10
IIS, age 1-2-3-5 y (IgE)	5.8 (1.1-30.5)	.038	0.5 (0.2-1.2)	.137	.01

OR, Odds ratio.

appeared to increase the risk of sensitization (Table III; Figs 1, *A,* and E1). Similarly, in IIS, in a longitudinal model including data from all 4 time points, children carrying a T allele were significantly less likely to develop sensitization if they attended day care ($P = .03$), although the interaction between day care and *TLR2/–16934* was not statistically significant ($P = .10$; Table III). Inspection of the patterns suggested that among children with an AA genotype, day care did not appear to have an effect on sensitization at ages 1 and 2 years, but there was a trend toward increase at ages 3 and 5 years among children who attended day care (Fig 1, *B*).

Data on current wheeze are presented in Tables E1, E2, and E3 and Figs E2 and E3 in the Online Repository at www.jacionline.org.

Atopic wheeze

In a longitudinal model in IIS including data from ages 1, 2, 3, and 5 years, we found a significant interaction between day care attendance and *TLR2/–16934* on the development atopic wheezing ($P = .01$; Table III), in that children with AA genotype who attended day care had higher risk of atopic wheezing than those who did not attend day care, whereas among T-allele carriers, day care attendance appeared protective. Although we observed a similar pattern in MAAS, the interaction between day care and genotype failed to reach statistical significance (Table III). In a longitudinal model of atopic wheezing (IgE) in the United Kingdom (UK) cohort, day care was associated with protection only among T-allele carriers ($P = .017$), whereas the direction of the association in children with AA genotype appeared to be in the opposite direction (Table III; see this article's Fig E4 in the Online Repository at www.jacionline.org). Inspection of the patterns (Fig 2, *A*) suggested that the interaction between day care attendance and *TLR2/–16934* was not evident before age 8 years (consistent with the finding that atopic wheeze at age 8 years was less common among children who attended day care; Table II).

Bronchial hyperresponsiveness (MAAS)

For the whole population, there was no association between day care and BHR at age 5 years. However, in children with T allele, day care was associated with less BHR, whereas among AA homozygotes, day care was associated with more BHR (Fig 3). The interaction between *TLR2/–16934* and day care was statistically significant (adjusted for baseline lung function; $P = .04$).

DISCUSSION

Key results

In 2 independent unselected birth cohorts from distinct geographic areas, we demonstrated that the association between day care attendance with sensitization and atopic wheezing appears dependent on a genetic variant in *TLR2*. Day care was protective, but only among children carrying the T allele for *TLR2/–16934*, whereas among AA homozygotes, there was no association between day care attendance and outcomes of interest, or the association tended to be in the opposite direction. In the MAAS cohort, socioeconomic status was significantly associated with day care attendance and some of the outcomes, but the significant interactions between day care and *TLR2/–16934* were maintained after adjusting for this factors with no material changes in the odds ratios for these models. These results were further strengthened by the similar findings for physiological measures strongly related to childhood asthma (dry air bronchial hyperreactivity) in one of the cohorts. Our results are consistent with those in children raised in a farming environment, where T allele carriers were less likely to have asthma and sensitization compared with AA homozygotes.[16] We postulate that attending day care and being raised on farm are markers of increased exposure to microbial products, which may have different or even opposite effects on asthma and allergies among carriers of different *TLR2/–16934* genotypes.

Limitations and strengths

We did not directly measure exposure to microbial agents but used day care attendance as a proxy. Several reports have shown that children attending day care centers experience more infections than children cared for at home,[19,20] and exposure to endotoxin (a component of the cell wall of gram-negative bacteria) is markedly higher in day care centers than in homes.[32]

The precise nature of the exposures in either the farming or day care environments nonetheless remains to be identified. Another limitation is that we relied on parental reports of wheezing, which may be unreliable because many parents have little understanding of what physicians mean by the term "wheeze."[33]

We made every effort to minimize false-positive results caused by multiple testing, but we acknowledge that we cannot fully eliminate the possible impact of multiple testing on the degree to which conclusions related to the statistical interactions can be considered reliable. The analysis was hypothesis-driven and limited to 1 genotype comparison in 2 carefully defined phenotypes. We minimized the number of phenotypes tested by capitalizing on the longitudinal nature of data collection and used longitudinal rather than a series of cross-sectional analyses.

We used slightly different definitions of day care attendance in the 2 populations. This is an inevitable consequence of the different provisions for maternity leave in the 2 countries, which influenced the age of entry to day care. In contrast with the

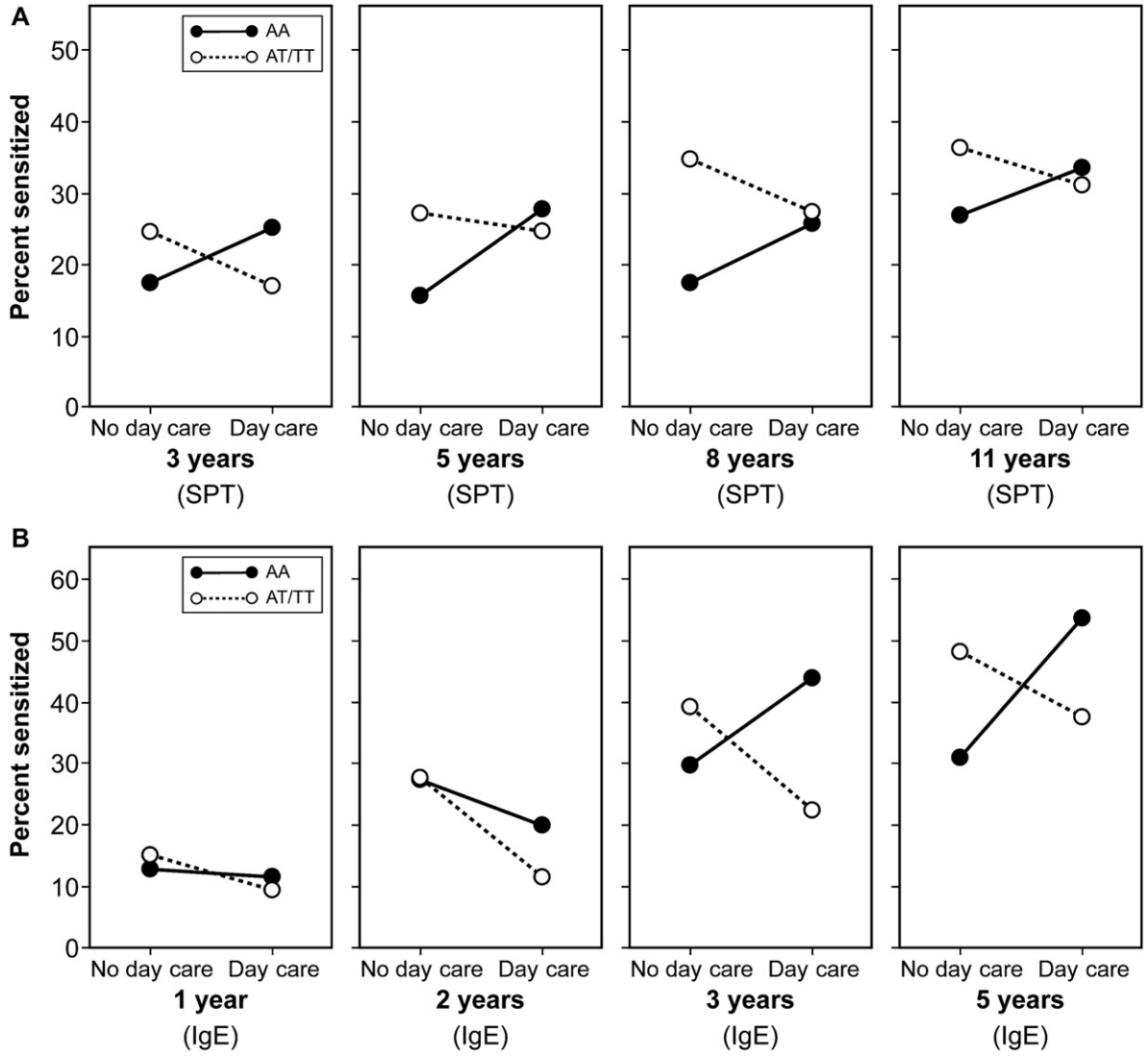

FIG 1. Percentage of children with allergic sensitization (assessed by SPT or specific IgE measurement *[IgE]*) by *TLR2/–16934* genotype and day care attendance in early childhood. **A,** MAAS. **B,** IIS.

United States, in the UK, paid maternity leave is provided for at least 9 months, and children are usually looked after by their mothers at home during this time (consequently, only 30 children in Manchester started nursery within the first 6 months of life, and we could not use more similar definitions of day care). It is worth noting that we used definitions of day care similar to those used in our previous studies demonstrating that in the whole populations, early day care exposure reduced IgE levels (IIS)[27] and reduced risk of wheezing (MAAS).[9] We therefore believe that our definitions of day care exposure in the 2 cohorts are appropriate for the distinct geographic areas and represent reasonable proxy measures of the exposure to infectious agents.

We acknowledge that the findings in 2 cohorts are not identical and that the interaction terms are either not significant or only marginally below the conventional .05 level. For example, the interaction between day care attendance and *TLR2/–16934* was significant for sensitization in MAAS and atopic wheeze in IIS but failed to reach statistical significance for atopic wheeze in MAAS and sensitization in IIS. Clearly, our conclusions would be stronger if the *P* values for interaction were all significant and if all were in the .001 range or below. However, even when the interaction did not reach statistical significance, all trends across different phenotypes in 2 populations were in the same direction. How does this compare with "replication" in studies of asthma and other complex diseases? Despite more than a decade of intensive work using a range of approaches from family-based linkage and candidate gene–based association studies to whole genome association studies, genetic studies have produced heterogeneous results with little replication.[3] It should be noted that in this context, "replication" refers to the finding of any association between the gene and any asthma or allergy phenotype. The gene is usually considered the unit of replication, reflecting the fact that frequently a different single nucleotide polymorphism (SNP) within the gene is a risk for disease, and sometimes even the opposite allele of the same SNP is the risk allele in different populations.[3] This phenomenon has been noted in most complex diseases; "precise" replication (ie, the same association of the same SNP with the same phenotype) is very rare.[34] Thus,

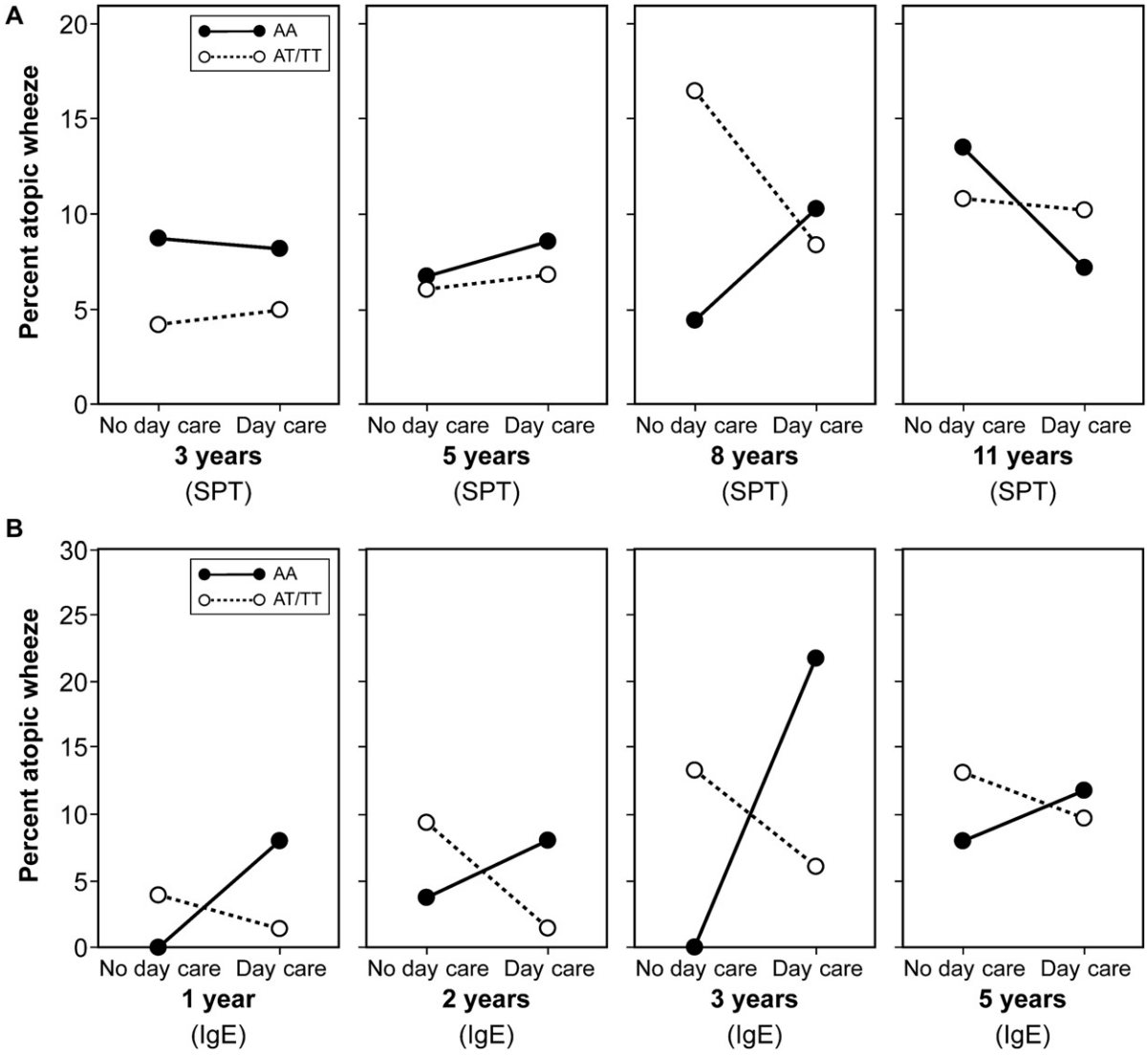

FIG 2. Percentage of children with atopic wheeze by *TLR2/–16934* genotype and day care attendance in early childhood; atopy was assessed by SPT or specific IgE measurement *(IgE)*. **A,** MAAS. **B,** IIS.

although we recognize that the findings in our 2 cohorts are not identical, it is reassuring that the direction of the interaction between the same SNP and similar environmental exposure across phenotypes in the 2 different populations was very similar.

Finally, we do not have the functional explanation for our findings. The *TLR2/–16934* polymorphism is a marker for a group of highly linked *TLR2* SNPs, and any of these SNPs may be responsible for the interaction described. We chose *TLR2/–16934* for these studies because it had been previously associated with asthma and allergies in farming environment.[16] TLR2 expression is increased in blood cells from children of farmers compared with children not raised on farms, suggesting that the innate immune system may respond to the microbial products present in the farming environment and may modulate the development of allergic disease.[35] Whether similar changes in TLR2 are present in children attending day care is unknown. TLR2 is the innate immune receptor for molecular patterns present on the surface of many microbial agents.[36] It is likely that expression of TLR2 on the cell surfaces is in part genetically determined, and this differential expression by genotype could modulate susceptibility to the effects of ligands present in microbial products.

A major strength of our studies is careful longitudinal phenotyping from birth in 2 unselected populations in distinct geographic areas. The phenotypic expression of asthma and allergic diseases starts early in life, and these phenotypes are unstable and may progress or remit over time. Thus, the optimal study design is a birth cohort because it overcomes problems of recall bias and permits longitudinal phenotyping and contemporaneous measurement of environmental exposures. This approach is crucial for the assessment of gene-environment interactions.

Interpretation

Published studies investigating the effect of day care on the development of allergic disease are inconsistent, with some showing increased risk[4] and others decreased risk[5-8] or no effect.[10,11] Similarly, polymorphisms in *TLRs* have been associated

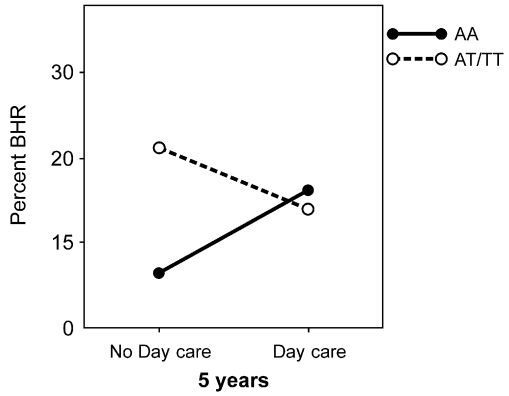

FIG 3. Percentage of children with BHR to EVH challenge at age 5 years by *TLR2/–16934* genotype and day care attendance in MAAS.

with allergic diseases in some[16] but not all studies.[18] These inconsistencies may be in part consequent to the differences in study designs, definitions of exposures and outcomes, or sample size. However, they may also reflect the fundamentally different nature of the relationship between genetic polymorphisms, environmental exposures, and phenotype in complex diseases compared with diseases determined predominantly by genetic factors. The relationship between genotype and phenotype in complex diseases may not be linear or unidirectional[37] but modulated by a number of environmental factors (for example, we have recently reported that cat ownership substantially increases the risk of early-life eczema in children with filaggrin loss-of-function variants, but not among those without).[38] Thus, the true associations between genetic variants and phenotype expression may be lost in studies in which study participants are exposed to a wide range of unmeasured environmental factors.[37] It is important to note that we found no association between *TLR2* genotype and clinical outcomes before we explored its interaction with day care attendance. The true significance of the genetic variant was uncovered only when the relevant environmental exposure was taken into account. Similarly, when we carried out the analysis in the whole population, day care appeared to be associated with a significant protection from atopic wheezing. However, this concealed the fact that among AA homozygotes, day care was not associated with protection but actually tended to increase the risk of atopic wheezing. The apparent protective effect in the whole population was consequent to the fact that children with a T allele (in whom day care was associated with less atopic wheezing) outnumbered AA homozygotes (in whom day care was associated with more atopic wheezing) by a factor of 3:1.

Recent studies in mouse models have strongly suggested that gene-environment interaction plays a crucial role in determining complex phenotypes. Valdar et al[39] reported the heritability of 88 complex traits that included models of human disease such as asthma and immunologic, biochemical, and hematologic phenotypes. They found that environmental covariates were involved in a large number of significant interactions with genetic background. Moreover, the effects of gene-environment interactions were more frequent and larger than the main effects: half of the interactions explained more than 20% of the variance of the complex phenotypes studied. It is thus plausible to surmise that the types of gene-environment interactions we have observed are not limited to the phenotypes we studied but may be crucial determinants of many other complex human phenotypes.

Generalizability

Our results suggest that in complex diseases such as asthma and allergies, genetic predisposition may need to be taken into account when assessing the effect of environmental exposures, and *vice versa*, relevant environmental exposures may need to be factored into the genetic association studies. Furthermore, we often use epidemiologic data to identify potentially modifiable risk factors to help devise primary prevention strategies. If we extrapolate our data to the context of primary prevention, the results suggest that only individuals with particular genotypes may benefit from a specific intervention, whereas the same intervention among individuals with different susceptibility may cause harm.

Our data indicate that the effects of day care on allergic phenotypes may differ among children with different variants of the *TLR2* gene. Children with T allele for *TLR2/–16934* may benefit from attending day care, whereas for those who are AA homozygotes, being cared for at home may prove beneficial. However, we emphasize that a caution is needed when interpreting our results because of marginal *P* values of the interaction terms and the fact that we cannot fully eliminate the multiple testing problem.

Clinical implications: Extrapolation of our data to the context of primary prevention suggests that only individuals with particular genotypes may benefit from a specific intervention, whereas the same intervention among individuals with different susceptibility may cause harm.

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METHODS

Study populations

MAAS. The MAAS is an unselected, population-based prospective study that follows the development of asthma and other atopic disorders in a cohort of children. The setting is the maternity catchment area of Wythenshawe and Stepping Hill Hospitals, composed of 50 square miles of South Manchester and Cheshire, UK, a stable mixed urban-rural population. The study was approved by the local research ethics committee. Informed consent was obtained from all parents.

Screening and recruitment. All pregnant women were screened for eligibility at booking antenatal visits (8th-10th week of pregnancy). The study was explained to the parents, and informed consent for initial questionnaires and SPT was obtained. Both parents completed a questionnaire about their and their partner's history of asthma and allergic diseases and smoking habits.

If the pregnant woman's partner was not present at the antenatal clinic visit, an invitation was sent for him to attend an open-access evening clinic for SPT and the questionnaire. Once both parents had completed questionnaires and SPT, a full explanation of the proposed future follow-up for the child was given.

Of the 1499 couples who met the inclusion criteria (≤10 weeks of pregnancy, maternal age ≥18 years, questionnaire and skin test data available for both parents), 288 declined to take part in the study. Of 1211 couples who initially agreed to take part, 1085 had a successful full-term pregnancy (>36 weeks of gestation) and gave consent to a further follow-up. Of those, 128 were prenatally randomized into an environmental control group, and 957 were followed as an observational arm of the cohort. We reviewed 1025 children at age 8 years; 122 randomized to an environmental intervention were excluded from this analysis. Samples for genotyping were provided by 727 children of mixed European ancestry, of whom 504 attended day care.

Follow-up. The children have been followed prospectively and attended review clinics at ages 3, 5, 8, and 11 years (±4 weeks).

Definitions of exposures and outcomes. Day care attendance: Day care data were derived from parental reports. Children were assigned into 2 categories according to the day care attendance: (1) no day care (ie, looked after at home or by child minder) and (2) regularly attended day care.

Atopic sensitization: Atopic sensitization was ascertained by SPT at ages 3, 5, 8, and 11 years (*Dermatophagoides pteronyssinus*, cat, dog, grasses, molds, milk, egg; Bayer, Elkhart, Ind). We defined sensitization as a mean weal diameter 3 mm greater than negative control to at least 1 of the allergens tested. We also measured specific serum IgE to mite, cat, dog, grasses, milk, egg, and peanut by ImmunoCAP (Phadia, Uppsala, Sweden) collected at 3, 5, and 8 years. The detection limit of the assay was 0.2 kU_A/L, and sensitization was defined by a specific IgE value >0.35 kU_A/L to at least 1 of the allergens tested.

Wheeze: A validated International Study of Asthma and Allergies in Childhood questionnaire was interviewer-administered to collect information on parentally reported symptoms, physician-diagnosed illnesses, and treatments received. Atopic wheeze was defined as a positive response to the question, "Has your child had wheezing or whistling in the chest in the last 12 months?" in the presence of atopic sensitization determined by skin testing or IgE measurement.

Lung function: At age 5 years, we carried out measurements of specific airway resistance (sR_{aw}) to assess airway function in all children who were willing to cooperate. Measurements of sR_{aw} were made using a constant volume whole body plethysmograph (Masterscreen Body 4.34; Jaeger, Würzburg, Germany). Flow and volume were measured with a heated differential pressure screen-type pneumotachograph with a resistance of 0.036 $kPa^{-1} \cdot s$ and a dead space of 160 mililiters. Pressure measurements were made with a pressure transducer (Nr.660.99007; Hube Control AG, Wuerenlos, Switzerland) with an input range of ±100 Pa, a resolution of 0.05 Pa, and a linear response up to 10 Hz. The plethysmograph was calibrated daily. Sensors in an ambient unit supplied with the plethysmograph recorded ambient data on temperature, humidity, and barometric pressure. The pneumotachograph was volume calibrated according to the American Thoracic Society recommendations by using a 2-L syringe at flow rates of 0 to 1.5, 1.5 to 5, and >5 L/s. The half value period was calibrated to ensure a specific leakage in the box of 4 to 7 seconds.

The pressure transducer was calibrated using a 50-mL motor driven piston pump to generate sinusoidal variations of plethysmographic pressure. Electronic body temperature, pressure, and saturation compensation was applied throughout by using a time shift of 60 ms.

sR_{aw} is measured by a single-step procedure from the simultaneously measured changes of respiratory flow and changes of plethysmographic pressure, omitting the measurement of thoracic gas volume. Measurements were carried out during tidal breathing using a facemask, which was adapted by fitting a standard pediatric facemask with a noncompressible mouthpiece made from silicone tubing. The end of the tubing was made rigid with an aluminum splint. The purpose of this was to maintain stable airway opening, prevent nose breathing, and support the cheeks. The procedure was explained to the accompanying adult and the use of the facemask demonstrated to the child. The children were encouraged to sit in the plethysmograph alone, but if they refused, the accompanying adult, usually a parent, accompanied the child in the plethysmograph cabinet with the child seated on the parent's knee. The door of the plethysmograph was closed and the subject asked to breathe through the facemask.

Children were encouraged to breathe at a rate of 30 to 45 breaths per minute. If a parent accompanied the child, the adult was asked to inhale and hold the breath for approximately 20 seconds. sR_{aw} measurements were made once a stable breathing pattern had been re-established. Once a stable breathing pattern was established, at least 3 measurements of sR_{aw} were performed, and each was calculated from the means of 5 consecutively measured technically acceptable loops (each child performed at least 15 loops). The median of these 3 measurements of effective sR_{aw} was used in the analysis. The measured values of sR_{aw} were corrected for the influence of the pneumotachograph screen and for the volume displacement caused by the subject (or subject + parent).

Children were asymptomatic at the time of assessment of lung function.

Airway reactivity: At the 5-year follow-up, airway reactivity was assessed by means of an EVH challenge. Subjects hyperventilated gas containing 21% O_2, 5% CO_2, and the remainder N_2 with a water content <10 mg/L for 6 minutes at a ventilation rate of 75% of maximum voluntary ventilation (estimated prechallenge as $FEV_1 \times 22.5$). sR_{aw} was measured at baseline and 2, 5, and 10 minutes postchallenge. The highest sR_{aw} value measured postchallenge was recorded. A subject was defined as having bronchial hyperresponsiveness to EVH challenge if the percent increase in sR_{aw} after challenge was larger than the 90th percentile of the increase for the reference subjects (skin test–negative, never-wheezing as reported in the questionnaires at age 5 years). Parents were asked to withhold from giving the children short-acting bronchodilators for 6 hours and long-acting bronchodilators for 12 hours before testing.

Genotyping. PCR primers and probes were designed by using the Sequenom iPLEX software provided by the manufacturer (Sequenom, San Diego, Calif).

PCR reaction consisted of 2.850 μL Nano-pure water, 0.625 μL (10× stock) PCR Buffer, 0.325 μL $MgCl_2$ (final concentration, 1.625 mmol/L), 0.1 μL deoxynucleotidetriphosphate mix (500 umol/L), 1 μL Primer mix (500 nmol/L of each), Genomic DNA (20 ng, dried down), 0.1 μL Hotstar Taq (Qiagen, Hilden, Germany; 0.5 U/reaction) in a final reaction volume of 5 μL. PCR amplification conditions were as follows: 94°C for 15 minutes; 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute; and a final extension step of 72°C for 3 minutes.

The PCR product was cleaned using a shrimp alkaline phosphatase (SAP) mixture to dephosphorylate the unincorporated dNTPs. SAP treatment mixes consisted of the following; 1.530 μL Nano-pure water, 0.170 μL 10× SAP buffer, and 0.300 μL SAP enzyme (1 U/μL) in a total reaction volume of 2 μL.

The probes were split into low and high mass and were used at 0.625 umol/L and 1.25 umol/L, respectively. The sample is denatured at 94 for an initial 30 seconds and then a further 5 seconds. Strands are annealed at 52 for 5 seconds and extended at 80 for 5 seconds. This annealing and extension cycle is repeated 4 more times for a total of 5 cycles. The program then starts again at the denaturation step of 5 seconds at 94. This larger cycle continues for a total of 40 times. A final extension is done at 72 for 3 minutes and then the sample is cooled to 4 degrees. SBE-PCR products were desalted by using 6 mg cleaning resin according to the manufacturer's instructions (Sequenom).

A nano-dispenser was used to dispense desalted SBE-PCR reaction products onto a 384-element Spectro CHIP bioarray (Sequenom). Each well contained 15 nL purified SBE-PCR product. Samples were then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

IIS. The IIS is a prospective nonselected birth cohort study of immune system maturation and its relation to the development of asthma and allergic disease in childhood. Participants were healthy children born to pregnant women who planned to obtain care for their newborns from collaborating pediatricians. The cohort includes 482 children, 219 of whom were excluded from the current analysis because they (1) had at least 1 parent of nonwhite ethnicity, (2) lacked data on day care or *TLR2/–1694* genotype, or (3) lacked data on at least 1 outcome (sensitization or wheeze at ages 1, 2, 3, or 5 years).

Definitions of exposures and outcomes. Day care: Day care was assessed by nurse-administered parent questionnaires at ages 1, 2, 3, 4, 6, and 9 months. Children were categorized as having attended day care if parents answered yes to the question, "Is your child cared for outside of your home on a regular basis?" on at least 1 of these 6 questionnaires.

Atopic sensitization: Specific IgE was measured for 6 inhalant allergens (*Alternaria*, *Dermatophagoides farinae*, Bermuda grass, careless weed, olive, and mulberry tree) and 2 foods (ovalbumin, β-lactoglobulin) at 1, 2, 3, and 5 years of age by using the Pharmacia AutoCAP assay (Pharmacia/Upjohn, Kalamazoo, Mich) before its discontinuation in 2006, and subsequently by using Immulite 2000 (Siemens Medical Solutions, Los Angeles, Calif). Samples analyzed on both instruments (n = 25) yielded a correlation coefficient of 0.995. The detection limit of the assay was 0.1 kU_A/L, and sensitization was defined as specific IgE value >0.35 kU_A/L to at least 1 of the allergens tested.

Genotyping. DNA samples were isolated from whole blood by using standard techniques. Genotyping of DNA samples was performed by using Taqman (Applied Biosystems, Carlsbad, Calif) assays.

RESULTS

Current wheeze

Tables E1 and E2 summarize the data on current wheezing overall, by *TLR2/–16934*, and by day care attendance in the 2 cohorts. There were no significant associations between *TLR2/–16934* and current wheezing in either cohort at any of the time points. In MAAS, day care was significantly associated with reduced wheeze at ages 5 and 8 years, and in IIS, day care was significantly associated with increased wheeze at age 1 year (Table E2).

Interaction between *TLR2/–16934* and day care

Current wheeze. The relationship between day care and wheeze did not appear to be modified by *TLR2/–16934* on the basis of assessment of interaction by longitudinal model in either cohort (Fig E2). However, in MAAS at age 5 and 8 years, the significant protective effect of day care on current wheezing was confined to T allele carriers and was not observed among children with AA genotype (age 5 years: AT + TT 25.9% vs 16.5% [$P = .011$], AA 29.8% vs 19.4% [$P = .157$]; age 8 years: AT + TT 24.5% vs 13.2% [$P = .001$], AA 14.6 vs 13.5 [$P = .852$]; home vs day care, respectively). Similarly, in IIS, day care was associated with protection in carriers of the T allele at ages 3 and 5 years (Fig E3).

Atopic sensitization assessed by IgE. Using atopy defined by IgE in MAAS did not materially alter the results, although formal statistical significance was not reached, probably because of a smaller sample size (eg, *TLR2/–16934*—day care attendance interaction, $P = .059$; Fig E1). However, it is worth noting that the interaction between *TLR2/–16934* and day care attendance on atopy (defined by IgE) was significant at age 8 years ($P = .016$), indicating a marked reduction in the risk among T allele carriers who attended the day care compared with those who did not.

Atopic wheeze. When we used IgE to define sensitization, day care attendance was associated with less atopic wheezing among children with T allele for *TLR2/–16934*, whereas we found an association in the opposite direction among children who were AA homozygotes (ie, day care attendance was associated with more atopic wheezing; Fig E4). As with atopy, the interaction between day care attendance and *TLR2/–16934* on atopic wheeze (defined by IgE) was significant at age 8 years ($P = .019$). This association remained unchanged when nonatopic nonwheezers were used as a reference group ($P = .010$).

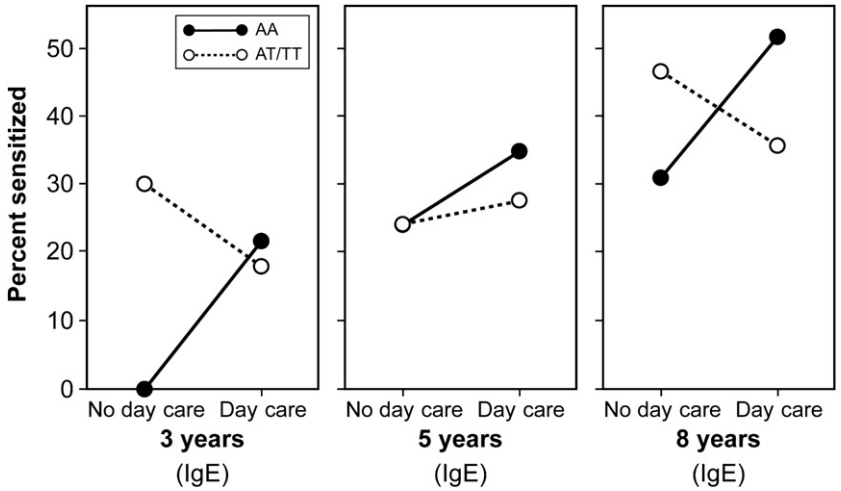

FIG E1. Percentage of children with allergic sensitization (assessed by specific IgE measurement) by *TLR2/–16934* genotype and day care attendance in early childhood in MAAS.

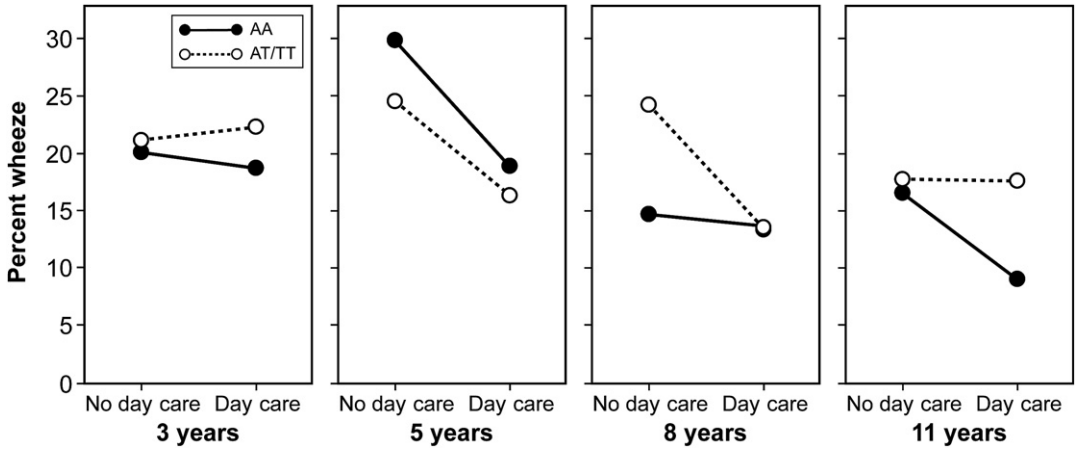

FIG E2. Percentage of children with current wheeze by *TLR2/–16934* genotype and day care attendance in early childhood in MAAS.

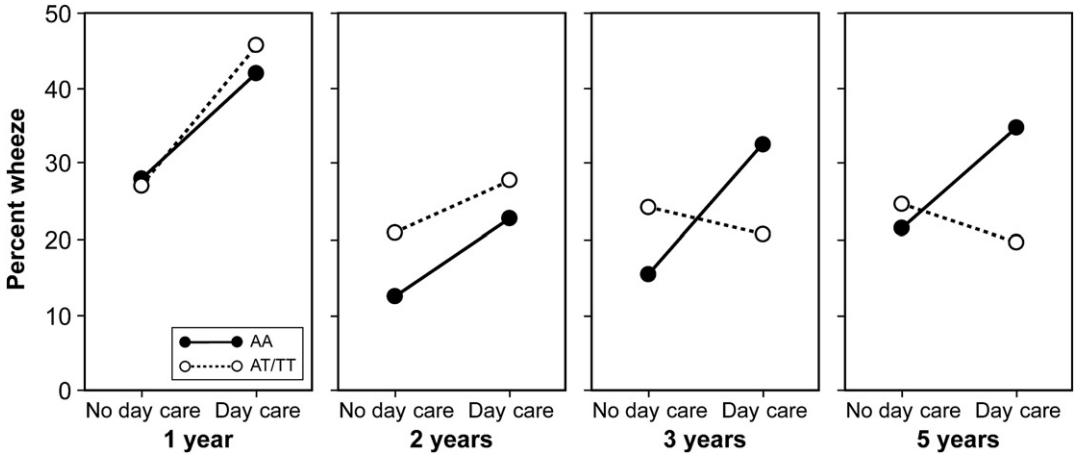

FIG E3. Percentage of children with current wheeze by *TLR2/–16934* genotype and day care attendance in early childhood in IIS.

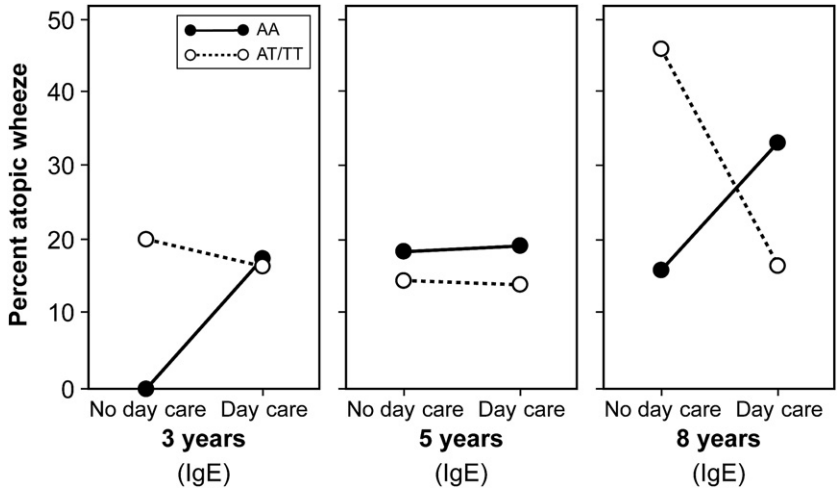

FIG E4. Percentage of children with atopic wheeze (sensitization assessed by specific IgE measurement) by *TLR2/–16934* genotype and day care attendance in early childhood in MAAS.

TABLE E1. Current wheezing by *TLR2/–16934* genotype

Variable	Whole group Frequency (%)	AA Frequency (%)	AT + TT Frequency (%)	*P* value
MAAS				
Age 3 y	157/709 (2.1)	30/157 (19.1)	127/552 (23.0)	.30
Age 5 y	143/703 (20.3)	35/155 (22.6)	108/548 (19.7)	.43
Age 8 y	112/697 (16.1)	21/152 (13.8)	91/545 (16.7)	.39
Age 11 y	104/637 (16.3)	16/142 (11.3)	88/495 (17.8)	.06
IIS				
Age 1 y	92/257 (35.8)	23/67 (34.3)	69/190 (36.3)	.88
Age 2 y	51/230 (22.2)	11/63 (17.5)	40/167 (24.0)	.37
Age 3 y	53/234 (22.6)	15/64 (23.4)	38/170 (22.4)	.86
Age 5 y	53/228 (23.2)	17/62 (27.4)	36/166 (21.7)	.38

TABLE E2. Current wheezing by day care attendance

Age	No day care Frequency (%)	Day care Frequency (%)	*P* value
MAAS			
Age 3 y	46/213 (21.6)	111/496 (22.4)	.82
Age 5 y	57/213 (26.8)	86/490 (17.6)	.005
Age 8 y	46/207 (22.2)	66/490 (13.5)	.004
Age 11 y	33/187 (17.7)	71/450 (15.8)	.561
IIS			
Age 1 y	36/132 (27.3)	56/125 (44.8)	.004
Age 2 y	22/119 (18.5)	29/111 (26.1)	.20
Age 3 y	26/120 (21.7)	27/114 (23.7)	.76
Age 5 y	27/115 (23.5)	26/113 (23.0)	1.00

TABLE E3. Odds ratios and CIs for the effect of day care on clinical outcomes in longitudinal models (unless stated otherwise), stratified by *TLR2/–16934*

	TLR2 = AA		TLR2 = AT/TT		
Variable	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**	**Interaction *P* value**
Current wheeze					
MAAS, age 3-5-8-11 y	0.7 (0.4-1.3)	.266	0.7 (0.5-1.0)	.062	.95
IIS, age 1-2-3-5 y	2.2 (1.0-4.8)	.046	1.3 (0.8-2.2)	.233	.29

OR, Odds ratio.

Toll-like receptor 2 as a major gene for asthma in children of European farmers

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Background: The finding that the prevalence of asthma and allergies is less frequent in children raised on animal farms has led to the conjecture that exposure to microbial products modifies immune responses. The toll-like receptors (TLRs) represent an evolutionarily conserved family of innate immunity receptors with microbial molecules as ligands.
Objectives: We reasoned that polymorphisms in genes encoding TLRs might modulate the protective effects observed in farming populations.
Methods: Farmers' and nonfarmers' children living in rural areas in Austria and Germany and who were enrolled in the cross-sectional ALEX study were genotyped for single nucleotide polymorphisms in the *TLR2* and *TLR4* genes. The frequencies of asthma, allergic rhinitis, and atopic sensitization were compared between the genotypes in relation to exposure to farming and endotoxin.
Results: Among farmers' children, those carrying a T allele in *TLR2/–16934* compared with children with genotype AA were significantly less likely to have a diagnosis of asthma (3% vs 13%, *P* = .012), current asthma symptoms (3% vs 16%, *P* = .004), atopic sensitization (14% vs 27%, *P* = .023), and current hay fever symptoms (3% vs 14%, *P* = .01). The association between *TLR2/–16934* and asthma among children of farmers was independent of atopy. No such association was found among children from the same rural communities but not living on farms.
Conclusion: Our results suggest that genetic variation in *TLR2* is a major determinant of the susceptibility to asthma and allergies in children of farmers. (J Allergy Clin Immunol 2004;113:482-8.)

Key words: *Farming, endotoxin, asthma, atopy, children, TLR2, TLR4, gene-environment interaction*

Abbreviations used
EU: Endotoxin units
OR: Odds ratio
TLR: Toll-like receptor

Asthma and allergies are complex diseases with a strong familial component suggestive of a genetic predisposition.[1,2] There have been numerous reports of an increase in the prevalence of asthma and allergies over the past decades in countries with a high socioeconomic status that cannot be explained by a change of the genetic background. Of interest in this regard are recent studies in Europe and North America pointing to the key role of a strong environmental influence because children and adults raised on animal farms were consistently found to have a lower prevalence of asthma, hay fever, and IgE-mediated reactivity to local allergens than those living away from farms.[3-9] Because a large variety of microbes is detectable on animal farms,[10] these findings are in line with the hypothesis that exposure to microbial products in early life modifies immune responses away from asthma and allergies.[11,12] In support of this contention, we have recently shown that exposure to bacterial endotoxin was inversely related to the frequency of atopic asthma, hay fever, and sensitization against common aeroallergens in school-aged children.[13]

Many bacteria have pattern molecules on their surfaces, and these molecules interact with pattern-recognition receptors, which are part of the innate immune system. Among the known pattern-recognition receptors for microbial products, toll-like receptors (TLRs) are an evolutionarily conserved group of molecules expressed in antigen-presenting cells and epithelial cells.[14] There are currently 10 different TLRs described in human subjects, and TLR2 and TLR4 have been studied most extensively in cell cultures and in animal models. In general, TLR2 has been found to be part of the signaling complex in response to a wide range of components of the cell membrane of gram-positive and gram-negative bacteria, mycoplasma, mycobacteria, parasites, and yeast,[15] whereas TLR4 seems to be the exclusive TLR for LPS, a major component of the outer cell membrane of gram-negative bacteria.[16] The genes encoding TLRs show a high variability in human populations,[17] but whether these genetic variations modify the interaction with microbial molecules and thereby modify the frequency of asthma and allergies is not yet known.

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We therefore reasoned that if microbial products are responsible for the lower prevalence of asthma and allergies among farmers' children and in children heavily exposed to endotoxin, then polymorphisms in *TLR2* and *TLR4* might modulate these effects. We tested this hypothesis in children from rural communities in Austria and Germany who were enrolled in the cross-sectional ALEX (Allergy and Endotoxin) study.[8]

METHODS

Population, phenotypes, and exposure to farming and endotoxin

Our study population included 609 children in school grades 1 to 6 from rural areas in Austria and Bavaria (Germany) who took part in the cross-sectional ALEX study, as previously described.[8] In brief, a self-administered questionnaire on respiratory and allergic diseases was filled out by the parents of children living on a farm and the parents of children living in the same rural area but not raised on a farm. Participating children and their parents were asked for consent to venipuncture and to collect a dust sample from their homes. The analyses in this study were restricted to children from Austria and Bavaria because samples for genotyping from the Swiss children were not available.

Asthma diagnosis was defined as a physician's diagnosis of asthma or recurrent asthmatic, obstructive, or spastic bronchitis. Children whose parents reported "wheezing or whistling in the chest in the last 12 months" were classified as having current asthma symptoms. Current hay fever symptoms were defined as a positive response to the following question: In the last 12 months, has your child had problems with sneezing or a runny or blocked nose without a cold accompanied by itchy-watery eyes? Atopy was defined as a specific IgE level of 3.5 kU/L or greater to at least one of the allergens tested. The concentration of specific IgE against grass pollen, birch pollen, house dust mite (*Dermatophagoides pteronyssinus*), cat dander, cow epithelium, and storage mite (*Lepidoglyphus destructor)* was measured in all sera (CAP, Pharmacia) in a central laboratory (University Children's Hospital Berlin Charity).

Farmers' children were defined as children whose parents answered yes to the following question: Do you live on a farm? For measurement of endotoxin, dust samples were taken from the child's mattress,[13] stored at room temperature, and shipped to a central laboratory (in Munich, Germany). The content of endotoxin in all dust samples was determined by using a kinetic Limulus assay (Bio Whittaker)[18] and expressed in endotoxin units (EU) per milligram. High endotoxin exposure was defined in children with endotoxin concentrations of greater than the median of the study group and compared with those less than the median.

Genotyping

As part of the National Heart, Lung, and Blood Institute Programs for Genomic Applications, we recently catalogued genetic variation in a central family of pattern-recognition receptors, the *TLR* genes. Details of the resequencing strategy and a full account of the results from 3 ethnic groups (white, African American, and Hispanic subjects), including linkage disequilibrium, main haplotypes found, and genotype frequencies, are available on the Programs for Genomic Applications Web site (http://innateimmunity.net).

Single nucleotide polymorphisms needed to identify haplotypes seen more than once in the screening process among white subjects and with a minor allele frequency of 10% or more were genotyped on the basis of the assumption that for polymorphisms with a lower minor allele frequency, power would be less than 0.50 to reject the hypothesis of no association with an α value of 0.05 (2-sided) in our sample. These single nucleotide polymorphisms were as follows: for *TLR2*, *TLR2/–16934*, *TLR2/+596*, and *TLR2/+1349*; for *TLR4*, *TLR4/–6143*, *TLR4/–5724*, *TLR4/+7263*, and *TLR4/+8469*. In addition, we genotyped the population for *TLR4/+4434* because it induces a change in the amino acid sequence of the protein, and this polymorphism is associated with hyporesponsiveness to inhaled endotoxin in human volunteers.[19]

DNA samples were isolated from whole blood by using standard techniques. DNA concentration was determined by using fluorescence spectroscopy (PicoGreen, Molecular Probes). Genotyping of DNA samples was performed with 5′-exonuclease (Taqman, Applied Biosystems) assays. Each assay was optimized for the PCR annealing temperature. Optimized genotyping reactions (10 μL of total volume, 0.9 μmol/L PCR primers, 0.2 μmol/L of each probe, 20 ng of genomic DNA, 1× master mix; Applied Biosystems) were set up in 384-well plates and thermal cycled in MJ tetrads (10 minutes at 95°C; 40 cycles of 15 seconds at 95°C; T_{an}, 1 minute). Taqman reactions were analyzed in a 7900HT (Applied Biosystems) instrument. Each 384-well plate contained 15 samples for which the genotype had been determined independently by means of DNA sequencing, as well as 4 no-DNA blank wells. Accuracy of genotypes (concordance of DNA sequencing derived genotypes to Taqman derived genotypes) was greater than 99.5%.

Oligonucleotide sequences used for genotyping are given in Table I.

Statistical analyses

The frequencies of the polymorphisms in the study population were calculated and compared with a population in Hardy-Weinberg equilibrium by using a χ^2 goodness-of-fit test. The same procedure was done in farmers' and nonfarmers' children separately.

The frequencies of asthma, current asthma symptoms, hay fever, and atopy were compared between the genotypes of the polymorphisms in *TLR2* and in *TLR4* and with respect to exposure to farming and exposure to endotoxin. The difference in proportions was calculated by using the χ^2 test and the Fisher exact test as appropriate. The Mantel-Haenszel common odds ratio (OR) for the association between genotype and phenotype was computed and tested for homogeneity across strata of exposure.

Logistic regression models were used to estimate the effect of the genotype when potential confounding factors were taken into account. The following variables were considered as potential confounders and included in the logistic regression model: study area (Germany/Austria), sex, age in years (continuous), maternal education (low/medium/high), and exposure to tobacco smoke at home (yes/no). We additionally included farming status (yes/no) as a potential confounder when estimating the effect of the polymorphisms in children stratified by endotoxin exposure (high/low) and vice versa.

We calculated the causative fraction, which was defined as the proportion of the disease that would not have occurred had the factor been absent from the population, as suggested by Miettinen,[20] and applied the following equation:

$$\text{Causative fraction} = ([\text{OR associated with the trait} - 1]/\text{OR associated with the trait}) \times \text{proportion of patients with the trait}$$

Analyses were carried out by chromosomes to test for the association between *TLR2* and *TLR4* haplotypes and the phenotypes under study.[21]

All analyses were performed with Stata/SE 8.0 software (Stata Corp).

This work was approved by the Institutional Review Board of the University of Arizona, by the Human Subject Committees of the

TABLE I. Oligonucleotide sequences used for genotyping

	Probe (VIC)	PCR-forward	
TLR4/–6143	ACTTAGCATACATAATATT	AAGTGCTTGGAGGATATTACAGTAGAACTA	
TLR4/–5724	TTCACCAACACTTATT	GTGATTACCACATTTTACAGACCAGAA	
TLR4/+4434	TCGATGGTATTATTG	GGCCTGTGCAATTTGACCAT	
TLR4/+7263	CATCCACTCTTCC	GTTTCCTGTTGGGCAATGCT	
TLR4/+8469	CAAATGCACACATCT	GGTGTTTCCATGTCTCATGTACTAGTG	
TLR2/–16934	TCTGGTGAGGGTCAT	TGGTTCTGGAGTCTGGGAAGTC	
TLR2/+596	AGATGACTTACATTCTG	CAGATCTACAGAGCTATGAGCCAAAA	
TLR2/+1349	CACGTGTAACAGGC	ATTTGAACTTATCCAGCACACGAAT	
	Probe (FAM)	**PCR-reverse**	**T_{an} (°C)**
TLR4/–6143	ACTTAGCATGCATAATA	GGAAAGTAGCAAGTGCAATGTAAGTTT	56
TLR4/–5724	CACCAACGCTTATT	CCACAAATGGTGTACAGGAGTTCTC	58
TLR4/+4434	CTCGATGATATTATTG	AGTCACACTCACCAGGGAAAATG	59
TLR4/+7263	AACATCCACTGTTCC	CATTAATTCCAGACACATTGTTTTCTC	52
TLR4/+8469	AAATGCGCACATCT	CCTGATAGGGATACATAGGGATATGTG	54.5
TLR2/–16934	ATCTGGAGAGGGTCAT	CTCACCATGTGATGCTTTCCAT	60
TLR2/+596	TGACTTACGTTCTGAATT	CATTCCACGGAACTTGTAACATCTAC	60
TLR2/+1349	CACAGCGTAACAGG	TCCAGTGTCTTGGGAATGCA	60

FAM, Carboxyfluorescein; T_{an}, annealing temperature.

TABLE II. Frequency of the genotypes in *TLR2* polymorphisms in farmers' children and in nonfarmers' children

	Farming	Nonfarming
TLR2/–16934	n = 237	n = 387
AA	23.2%	25.6%
AT	50.2%	49.9%
TT	26.6%	24.5%
χ^2 test	$P > .2$	
TLR2/+596	n = 237	n = 386
CC	16.9%	18.1%
CT	52.7%	48.7%
TT	30.4%	33.2%
χ^2 test	$P > .2$	
TLR2/+1349	n = 239	n = 386
TT	90.0%	88.1%
CT	10.0%	11.4%
CC	0.0%	0.5%
Fisher exact test	$P > .2$	

Children's Hospital, by the University of Munich, and by the Children's Hospital, Salzburg, Austria. The nature and possible consequences of the studies were explained to the parents of the children involved, who provided written informed consent for this study.

RESULTS

Our study population included 229 farmers' children and 380 children from the same rural area but not living on a farm. The mean age was 9.4 years (SD, 1.5) in farmers' children and 9.3 years (SD, 1.5) in nonfarmers' children. There were slightly more boys among the farmers' children (56%) than among the nonfarmers' children (51%).

The median of the endotoxin concentrations from the mattress samples was 27.4 EU/mg dust (interquartile range, 24.0 EU/mg) and, as previously reported,[13] was significantly higher in farmers' children than in nonfarmers' children (37.6 EU/mg [interquartile range, 27.2 EU/mg] and 23.0 EU/mg [interquartile range, 17.5 EU/mg], respectively, $P < .0005$). Sixty-eight percent (156) of the farmers' children and 39% (149) of the nonfarmers' children were classified as heavily exposed to endotoxin (median or greater). In the high-exposure group the median of the endotoxin concentration was 41.3 EU/mg (interquartile range, 21.8 EU/mg), and in the low-exposure group the median of the endotoxin concentration was 17.4 EU/mg (interquartile range, 9.0 EU/mg). The association between endotoxin and asthma, hay fever, and atopy in the ALEX study population are described in detail elsewhere.[13]

The allele frequencies for all single nucleotide polymorphisms were not significantly different between farmers and nonfarmers, and none of them showed marked deviations from a population in Hardy-Weinberg equilibrium (Tables II and III). No difference in exposure to endotoxin was found between the genotypes of the polymorphisms in *TLR2* and *TLR4*.

TLR2 polymorphisms

Among farmers' children there was a strong association between the *TLR2/–16934* polymorphism and the phenotypes under study. Only those children carrying 1 or 2 T alleles in *TLR2/–16934* were significantly less likely to have a diagnosis of asthma, current asthma symptoms, atopy, and current hay fever symptoms (Fig 1, *A*). No such association was found in nonfarmers' children (Fig 1, *B*). The ORs for the association between the *TLR2/–16934* polymorphism and the phenotype were different between farmers' and nonfarmers' children for asthma (test for homogeneity for the ORs, $P = .07$), current asthma symptoms ($P = .008$), atopy ($P = .005$), and current hay fever symptoms ($P = .007$).

Logistic regressions were performed to determine the potential role of confounders in these results. For *TLR2/–16934*, associations remained significant for all

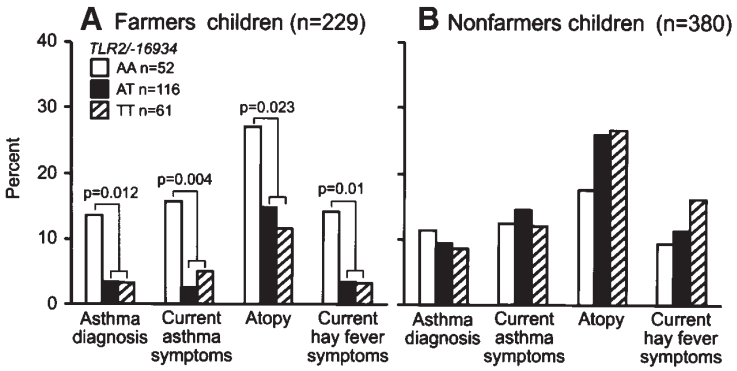

FIG 1. Prevalence of asthma, atopy, and current hay fever symptoms in *TLR2/–16934* in farmers' children **(A)** and in nonfarmers' children **(B)**.

phenotypes under study in the case of farmers and remained nonsignificant for the same phenotypes among nonfarmers (Table IV). When, in models with asthma as a dependent variable, specific IgE against any of the allergens tested was also included in the model together with the other confounders, the association remained significant for asthma diagnosis (OR, 0.18; 95% CI, 0.04-0.79; P = .02) and for current asthma symptoms (OR, 0.16; 95% CI, 0.05-0.58; P = .005). In farmers the causative fraction that is attributable to the genotype AA in *TLR2/–16934* was 44% for asthma diagnosis, 47% for current asthma symptoms, 21% for atopy, and 41% for current hay fever symptoms.

We next assessed the association between *TLR2/–16934* and asthma and allergies separately for subjects heavily exposed (≥50th percentile) and for those not heavily exposed to endotoxin. No clear association between genotypes and phenotypes was observed for either subjects heavily exposed to endotoxin or for subjects not heavily exposed (Table IV).

We found no association between either *TLR2/+596* or *TLR2/+1349* and any of the phenotypes under study for either farmers or nonfarmers or in relationship to endotoxin, and no additional information was obtained by using *TLR2* haplotypes (data not shown).

TLR4 polymorphisms

There was no statistically significant association between the *TLR4/+4434* polymorphisms and either asthma, allergic rhinitis, or atopy when the data were stratified by farming status (Table IV). However, when the data were stratified by level of exposure to endotoxin, we found that among children who were heavily exposed, *TLR4/+4434* showed a significant association with atopy (Fig 2, *A*), and a trend in the opposite direction was seen in children not heavily exposed (Fig 2, *B*). The Mantel-Haenzel test for homogeneity for the ORs across strata of endotoxin exposure was highly significant (P = .014). The association between the polymorphism and atopy remained significant after controlling for potential confounders (Table IV). No clear association was seen for asthma, current asthma symptoms, and current hay fever symptoms (Fig 2 and Table IV). Very similar results were obtained when endotoxin exposure was assessed as a continuous variable (data not shown).

No associations were seen for the other *TLR4* polymorphisms, and no additional information was obtained with *TLR4* haplotypes (data not shown). No significant gene-gene interaction was found between the *TLR4* and *TLR2* single nucleotide polymorphisms (data not shown).

TABLE III. Frequency of the genotypes in *TLR4* polymorphisms in farmers' children and in nonfarmers' children

	Farming	Nonfarming
TLR4/–6143	n = 231	n = 385
AA	46.3%	44.9%
AG	41.1%	45.7%
GG	12.6%	9.4%
χ^2 test	$P > .2$	
TLR4/–5724	n = 240	n = 396
TT	71.3%	75.5%
CT	25.4%	22.0%
CC	3.3%	2.5%
χ^2 test	$P > .2$	
TLR4/+4434	n = 236	n = 395
AA	88.1%	85.1%
AG	11.9%	14.2%
GG	0.0%	0.8%
Fisher exact test	$P > .2$	
TLR4/+7263	n = 241	n = 395
GG	71.0%	72.9%
CG	26.6%	26.3%
CC	2.5%	0.8%
Fisher exact test	$P > .2$	
TLR4/+8469	n = 242	n = 395
TT	76.4%	74.9%
CT	20.2%	23.3%
CC	3.3%	1.8%
χ^2 test	$P > .2$	

DISCUSSION

In this study we found that a polymorphism in the *TLR2* gene is strongly associated with the frequency of asthma and allergies in children of European farmers and that a

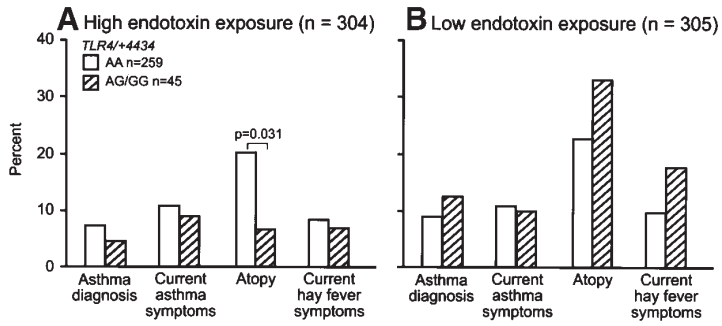

FIG 2. Prevalence of asthma, atopy, and current hay fever symptoms in *TLR4/+4434* in children exposed to high (≥50th percentile; **A)** and children exposed to low (<50th percentile; **B)** endotoxin concentrations (in EUs per milligram).

Mechanisms of allergy

TABLE IV. ORs, adjusted ORs,* and 95% CIs for asthma, atopy, and current hay fever symptoms in farmers' and in nonfarmers' children and in children exposed to high (≥50th percentile) and low (<50th percentile) endotoxin concentrations (in EUs per milligram) in relation to *TLR2/–16934* (AA, n = 149; AT/TT, n = 460) and *TLR4/+4434* (AA, n = 525; AG/GG, n = 84) polymorphisms

	Farmers (n = 229)		Nonfarmers (n = 380)	
	OR (95% CI)	**aOR† (95% CI)**	**OR (95% CI)**	**aOR† (95% CI)**
TLR2/–16934				
Asthma diagnosis	0.23 (0.07-0.70)	0.17 (0.04-0.77)	0.79 (0.38-1.67)	0.71 (0.33-1.53)
Current asthma symptoms	0.19 (0.06-0.57)	0.18 (0.05-0.61)	1.12 (0.56-2.24)	1.07 (0.53-2.17)
Atopy	0.43 (0.20-0.90)	0.41 (0.18-0.91)	1.67 (0.93-3.00)	1.60 (0.86-2.98)
Current hay fever symptoms	0.22 (0.07-0.67)	0.24 (0.07-0.84)	1.42 (0.66-3.07)	1.47 (0.65-3.33)
TLR4/+4434				
Asthma diagnosis	0.67 (0.08-5.36)	1.73 (0.17-17.19)	1.06 (0.42-2.66)	1.09 (0.43-2.77)
Current asthma symptoms	§		1.03 (0.46-2.32)	1.07 (0.47-2.46)
Atopy	0.19 (0.02-1.43)	0.22 (0.03-1.72)	1.10 (0.58-2.08)	1.09 (0.55-2.16)
Current hay fever symptoms	0.66 (0.08-5.30)	0.96 (0.11-8.43)	1.42 (0.65-3.14)	1.29 (0.56-2.97)
	Endotoxin: high (n = 304)		**Endotoxin: low (n = 305)**	
	OR (95% CI)	**aOR‡ (95% CI)**	**OR (95% CI)**	**aOR‡ (95% CI)**
TLR2/–16934				
Asthma diagnosis	0.61 (0.23-1.56)	0.72 (0.26-2.01)	0.51 (0.23-1.13)	0.42 (0.18-1.00)
Current asthma symptoms	0.67 (0.30-1.48)	0.72 (0.31-1.71)	0.70 (0.32-1.56)	0.70 (0.30-1.63)
Atopy	0.65 (0.34-1.23)	0.72 (0.37-1.43)	1.58 (0.83-3.03)	1.46 (0.73-2.93)
Current hay fever symptoms	0.63 (0.26-1.53)	0.93 (0.34-2.53)	1.03 (0.44-2.38)	0.89 (0.36-2.19)
TLR4/+4434				
Asthma diagnosis	0.59 (0.13-2.62)	0.54 (0.12-2.51)	1.48 (0.53-4.13)	1.46 (0.50-4.27)
Current asthma symptoms	0.80 (0.27-2.41)	0.76 (0.24-2.39)	0.96 (0.32-2.89)	0.89 (0.29-2.78)
Atopy	0.29 (0.09-0.96)	0.17 (0.04-0.75)	1.67 (0.81-3.45)	1.93 (0.89-4.19)
Current hay fever symptoms	0.78 (0.22-2.72)	0.55 (0.12-2.57)	1.98 (0.79-4.92)	2.06 (0.79-5.37)

aOR, Adjusted OR.
*Logistic regression was used to adjust for study area, sex, age, maternal education, and exposure to tobacco smoke at home; reference genotype was AA for *TLR2* and AA for *TLR4*, respectively.
†Additionally adjusted for current endotoxin exposure.
‡Additionally adjusted for farming.
§No child with current asthma symptoms in genotype AG/GG.

polymorphism in the *TLR4* gene has an influence on atopy in children heavily exposed to endotoxin. These results point to a gene-by-environment interaction because both *TLR2* and *TLR4* are genes encoding ancient pattern-recognition receptors for different microbial molecules.

The potential role of TLRs as regulators of immune responses has received considerable attention in recent years, and TLR activation in different types of cells is associated with the release of effector molecules, such as cytokines and chemokines,[22] that can influence adaptive immune responses. TLRs are present on the surfaces of antigen-presenting cells, regulatory T cells, and other cells that play crucial roles in the interface between environmental exposures and the immune system.[23,24] Sig-

nals produced by these cells control the maturation of both $CD4^+$ and $CD8^+$ T cells, and this maturation determines the type of adaptive immune response against environmental antigens. It was thus plausible to surmise that genetic variations in TLRs might have an influence in the pattern of immune responses that are established against environmental antigens.

In our study we found that a genetic variation in *TLR2 (TLR2/–16934)* is a major determinant of susceptibility to asthma and atopy in farmers' children. That microbial products present in a farming environment might interact with TLR2 is supported by the finding of an increased expression of this receptor in blood cells obtained from children of farmers compared with children not raised on farms.[25] It is not possible to deduce from our study the molecular mechanisms involved in the interaction between polymorphisms in *TLR2* and exposures present in children raised on farms because the functional significance of this polymorphism is not known. Moreover, other polymorphisms that are in linkage disequilibrium with *TLR2/–16934* could be responsible for the observed associations. However, if *TLR2/–16934* were the polymorphism mainly responsible for the observed effects, carriers of the T allele could be less likely to have asthma and allergies as a result of an increased expression of TLR2 on the surface of antigen-presenting cells or other cells. This, in turn, could increase their susceptibility to the protective effects of TLR2 ligands present in microbial products. It is not known whether a specific microbial molecule is essential for the observed association between asthma and the *TLR2* polymorphism in farmers' children or if activation of a common intracellular signaling pathway by any of the TLR2 ligands is sufficient. TLR2 interacts with components of a variety of gram-positive and gram-negative bacteria, mycobacteria, yeast, and parasites,[26-29] which are likely to be abundant in an animal-farming environment, but the design of this study does not allow us to identify the specific pattern-recognition molecules underlying our observed association.

In this study we also found that a genetic variation in *TLR4/+4434* is inversely associated with specific IgE to common aeroallergens in children heavily exposed to endotoxin. This is of interest because TLR4 is the exclusive TLR for LPS, the purified endotoxin from gram-negative bacteria, and *TLR4/+4434* leads to an amino acid change that alters the extracellular domain of the TLR4 receptor.[19] In recent studies, carrying the minor G allele for *TLR4/+4434* was associated with bronchial hyporesponsiveness to inhaled LPS in healthy volunteers.[19] In German adults only subjects with the minor G allele in *TLR4/+4434* showed an inverse association between exposure to house dust endotoxin and bronchial responsiveness to methacholine.[30] In our study population we previously found that exposure to indoor endotoxin has protective effects against the development of atopy and atopy-related phenotypes,[13] and we speculate that responsiveness to endotoxin in carriers of the minor G allele in *TLR4/+4434* explains, at least in part, the observed inverse association. Despite a strong association between the *TLR4/+4434* polymorphism and specific IgE in subjects heavily exposed to endotoxin, no clear association was seen between *TLR4/+4434* and either asthma or hay fever in these same subjects. A limited number of carriers of the minor G allele in *TLR4/+4434* in our data set could be one explanation for this discrepancy. Nevertheless, the role in the genetics of asthma and allergies of variations in other molecules (apart from TLR4) involved in the endotoxin receptor and signaling mechanisms is only beginning to be understood.[31]

The results of our study further suggest that the genetic and environmental mechanisms that determine asthma might be different from those determining atopy, despite a strong association between these 2 conditions in the population as a whole. Indoor endotoxin appears to modify the production of specific IgE and, by this mechanism, has a protective effect against hay fever and the atopic form of asthma, but it does not seem to have a direct protective effect against the development of nonatopic asthma.[13] On the other hand, a farming environment is associated with a lower prevalence of both atopic and nonatopic asthma,[8] suggesting that microbial products other than endotoxin play an important role in the association between exposure to animal farms and asthma. Our finding of an association between the *TLR2/–16934* polymorphism and asthma independent of atopy in farmers' children supports this conclusion.

The observed associations in farmers' children are unlikely due to population stratification. We found the associations in Austrian and in German farmers' children, and in both countries there were no such associations among children of nonfarmers. However, replication of our results in an independent study is clearly needed. We cannot exclude the possibility, for example, that avoidance of exposure to TLR2 ligands and other microbial products might have occurred in homozygotes for the A allele in *TLR2/–16934*, thus making them more susceptible to asthma and allergies among farmers' children. Further studies are needed to identify the TLR2 ligands responsible for the observed association to exclude the possibility that preferential avoidance of certain exposures might explain our findings.

Identification of products present in the environment that are responsible for the decreased risk of asthma in farmers' children might prove useful for future strategies for the primary prevention of asthma. Exposure to innocuous surrogates of these molecules at crucial times during the development of asthma-related immune responses in susceptible individuals could be associated with a marked decrease in the incidence of these diseases in much the same way as natural exposure to microbial products among children of farmers.

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